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MINIREVIEW

Laboratory Diagnosis of Lassa Fever

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ABSTRACT Lassa virus remains an important cause of illness in West Africa and among the travelers returning from this region with an acute febrile illness. The symptoms of Lassa fever can be nonspecific and mimic those of other endemic infections, especially early in illness, making a clinical diagnosis difficult; therefore, laboratory testing is needed to confirm the diagnosis. An early identification of Lassa fever is crucial for maximizing the benefit of available antiviral therapy, as treatment efficacy rapidly decreases following the clinical onset of the disease. This minireview provides an overview of the currently available diagnostic tests for Lassa fever and their strengths and weaknesses.

KEYWORDS Lassa fever

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Lassa virus, an arenavirus first isolated in 1969 in Jos, Nigeria (1), is the cause of Lassa fever, an acute viral illness that affects 100,000 to 300,000 persons per year based on 1970s estimates (2). Lassa fever is endemic in regions of West Africa, including Guinea, Liberia, Nigeria, and Sierra Leone, but cases have been exported to other countries by infected travelers. The natural reservoir for Lassa virus is the African soft-furred rat (*Mastomys natalensis*), which may be found throughout West Africa. The virus is transmitted to humans via direct contact with or the inhalation or ingestion of infected rat excreta or person to person via contact with infected body secretions (3).

Lassa fever presents with nonspecific symptoms similar to many other endemic illnesses in West Africa, making it difficult to diagnose clinically; therefore, laboratory testing is needed to confirm the diagnosis (4). The availability of laboratory testing has been limited by the designation of Lassa virus as a category A pathogen by the National Institute of Allergy and Infectious Diseases (5). Biosafety level 4 (BSL-4) precautions are recommended for handling potentially infectious specimens (6). In 2014, the World Health Organization issued a call for early diagnostic tests for Lassa fever (7). This article provides a brief review of the challenges of identifying Lassa fever and the different diagnostic tests available for Lassa fever along with their strengths and weaknesses.

CLINICAL PRESENTATION AND TREATMENT

Illness in humans develops within 3 weeks after infection with Lassa virus (3, 4). The initial symptoms of Lassa fever are nonspecific and may include fever, malaise, headache, sore throat, myalgia, cough, chest pain, abdominal pain, nausea, vomiting, and diarrhea (4, 8, 9). In most cases, symptoms are mild; however, severe illness complicated by abnormal bleeding, generalized edema, respiratory distress, hypotension, proteinuria, transaminitis, deafness, encephalopathy, and/or hypotension develops in approximately 20% of cases (3, 4, 8, 9). Although the overall fatality rate from Lassa fever is low (2, 10), it is 15 to 20% among patients who are hospitalized (3, 11). Higher fatality rates have been reported during outbreaks and among pregnant women, particularly in the third trimester of pregnancy (12). Treatment with ribavirin lowers the fatality risk to less than 5% if started in patients during the first 6 days of illness, but the beneficial effect on fatality is diminished if ribavirin is started later in the course of illness (13).

LASSA VIRUS DIAGNOSTIC CHALLENGES

One significant challenge in West Africa is differentiating between etiologies of febrile illness with similar initial clinical presentations, including malaria, influenza, dengue, yellow fever, and Lassa fever, with limited laboratory facility and reagent availabilities. Empirical treatment for presumed malaria or bacterial infection is often trialed, and Lassa fever is only suspected when a patient fails to improve with antimalarial and antibiotic therapy. This diagnostic delay leads to delayed patient isolation, an increased potential for transmission to family members and health care workers, and delayed initiation of ribavirin therapy, thereby decreasing its beneficial effect. Further highlighting the challenges of appropriate diagnostics is the emergence of Ebola virus in West Africa. A recent study found 60 to 70% of the patients with blood samples submitted to the Lassa Diagnostic Laboratory in Kenema, Sierra Leone, in the years prior to the Ebola virus outbreak were negative for malaria and Lassa virus, and there was serological evidence of Ebola and Marburg virus infections (14, 15). Correctly identifying the cause of an acute febrile illness in West Africa in an actionable time frame requires validated, rapid region-appropriate diagnostic assays.

Given the risk of person-to-person virus spread via bodily fluids, laboratory staff should be aware of the risk of Lassa virus when processing potentially infectious specimens. Poor sample storage and handling may pose a safety hazard to laboratory staff as well as decrease the sensitivity of diagnostic assays. The World Health Organization guidelines for the collection, storage, and handling of specimens for Ebola virus testing should be followed when testing for Lassa virus (16–18). BSL-4 precautions are recommended when handling specimens which may contain infectious Lassa virus (6); however, the availability of such high-containment laboratories is limited worldwide. If BSL-4 precautions are not available, samples may be handled in a class II or III biosafety cabinet or inactivated to allow safe handling of specimens under BSL-2 precautions (16, 19).

While there are multiple methods for viral inactivation in the literature, different methods are appropriate depending on the intended downstream testing (e.g., molecular or immunological pathogen detection, clinical laboratory tests, etc.). Chemical inactivation using solutions containing guanidine salts (e.g., TRIzol, Triton X-100, and buffer AVL combined with ethanol) is well documented, is effective with multiple pathogens, and is commonly used (20–22). Inactivation can be achieved by heating a blood specimen to 60°C for 60 min (23), although inactivation at 56°C for 30 min has been reported (24). Depending on the sample matrix and the specific pathogen, heat exposure alone may not result in complete inactivation; the use of chemical denaturizing solutions in combination with heating to provide more complete inactivation is recommended (19). Gamma irradiation is also used to inactivate Lassa virus in liquid and dried samples (23, 25–27). Since the required absorbed radiation dose for successful viral inactivation varies depending on the temperature of the sample (25), empirical sample safety testing is required to confirm inactivation.

The high-containment safety requirements complicate Lassa virus assay development and validation studies. Many assay reagents need to be generated under BSL-4 conditions. Synthetic nucleic acids and recombinant proteins are more commonly being used as assay components, but assay validation with mock clinical samples still requires viral materials generated under BSL-4 conditions.

The development of appropriate diagnostic assays is further complicated by significant Lassa virus diversity. The high nucleotide and amino acid diversity of Lassa virus isolates sequenced across West Africa (Fig. 1) can result in false-negative results if the primer/probe or antibody pairs do not bind to the target sufficiently. For example, a commonly used reverse transcriptase PCR (RT-PCR) assay (28) was redesigned when false negatives were identified due to primer-template mismatches (29). Furthermore, an NCBI protein BLAST analysis of the Lassa virus Josiah strain showed that glycoprotein ([GPC] NP_694870) and nucleoprotein ([NP] NP_694869.1) varied in percent identity from 91 to 99% and 86 to 99%, respectively, with full-length protein sequences of the

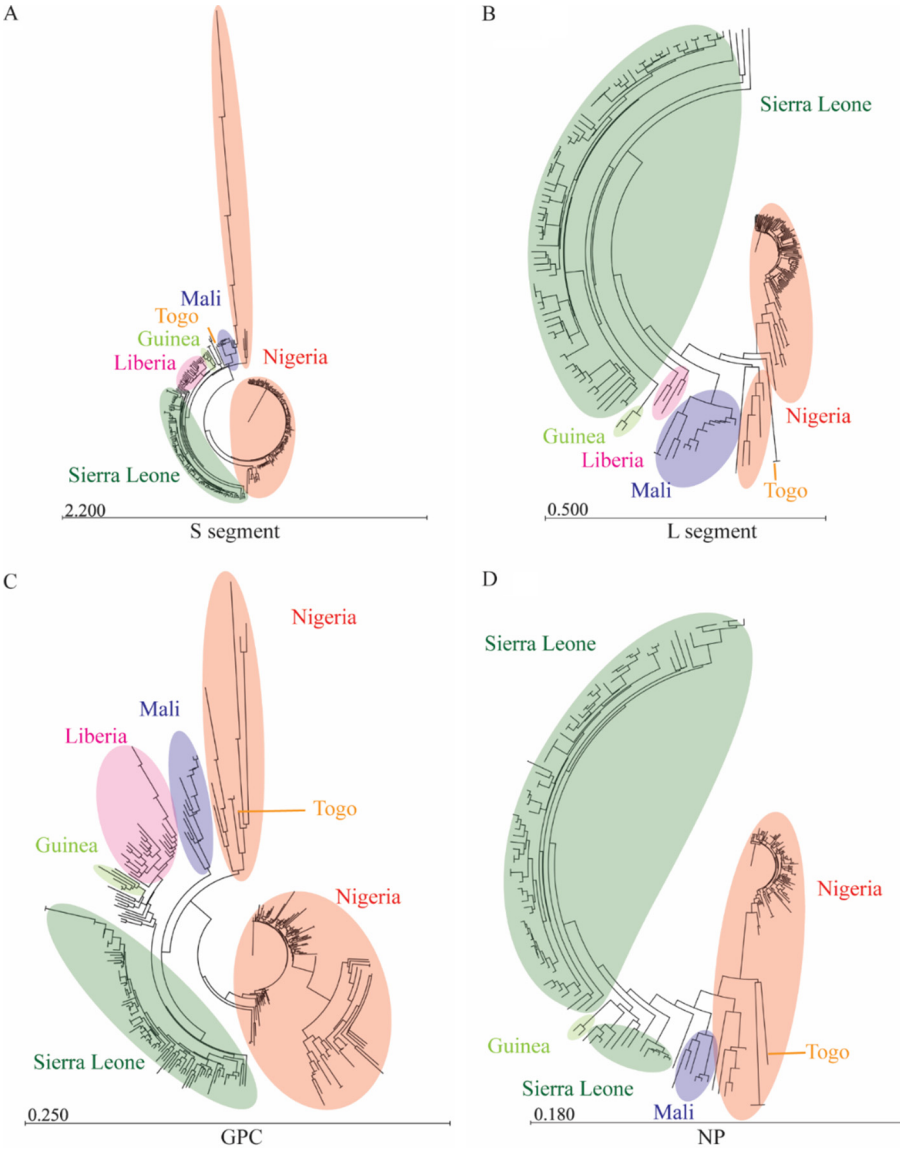

FIG 1 Lassa virus sequence diversity complicates efficacious assay design. Full and partial Lassa virus nucleic acid sequences were acquired from GenBank for the S RNA segment (A) and L RNA segment (B), and amino acid sequences for glycoprotein (GPC) (C) and nucleoprotein (NP) (D) were generated. Sequences were aligned, and phylogenetic trees (neighbor joining, Jukes-Cantor) were generated using CLC Genomics Workbench. Highlighted sections illustrate the geographic distribution and variability of Lassa virus.

other Lassa virus protein sequences in GenBank (30). For example, Emmerich and colleagues evaluated the anti-Lassa virus antibody response in a human sample set from West Africa by immunofluorescence assay (IFA) and reverse enzyme-linked immunosorbent assays (ELISAs) using several different Lassa virus strains (31). The authors found differing antibody responses depending on the virus strain used; stronger antibody responses were observed with local Lassa virus strains (31). Reassortant virus ML29, containing the L RNA segment of Mopeia virus and the S RNA segment of Lassa virus (Josiah strain), provides protection when injected into guinea pigs against distantly related strains of Lassa virus from Nigeria; ML29 could potentially serve as a broadly cross-reacting reagent for assay development (32, 33). Table 1 highlights a selection of Lassa virus assays found in the literature.

However, we are currently unaware of any Lassa virus diagnostic validation studies demonstrating assay performance using viruses isolated across West Africa that cover the wide diversity of Lassa genetic variation possible. In the absence of a single, timely,

TABLE 1 Selected assays for detecting Lassa virus

First author or assay	Assay type	Target[b]	Year	Reference
Demby et al.	Standard RT-PCR	5′-UTR/GPC	1994	28
Ölschläger et al.	Standard RT-PCR	5′-UTR/GPC	2010	29
Ölschläger et al.	RT-PCR/hybridization array	5′-UTR/GPC	2012	67
Drosten et al.	SYBR green real-time RT-PCR	5′-UTR/GPC	2002	41
Safronetz et al.	SYBR green real-time RT-PCR	GPC	2010	45
Trombley et al.	Real-time RT-PCR[a]	NP, GPC	2010	44
Bausch et al.	Ag (NP) and IgM capture, IgG ELISA	Irradiated virus	2000	36
Saijo et al.	Ag capture, IgG ELISA	rNP	2001	56
ReLASV antigen rapid test	Ag capture/LFI	rNP	2013	58, 59
Satterly et al.	Ag and IgM capture (MAGPIX)	rGPC, rNP	2016	53
O'Hearn et al.	IgG (MAGPIX)	Irradiated virus	2016	14

[a]Multiple assays are used to detect different Lassa strains.
[b]rNP, recombinant nucleoprotein; rGPC, recombinant glycoprotein complex.

pan-Lassa virus diagnostic assay, one future strategy could be the designing and validating of assays based on geographic region, as Lassa virus diversity generally clusters with geographic location (Fig. 1) (34). While ideal for use in specific countries/regions, this approach, in the context of exported cases of Lassa fever from multiple countries where it is endemic, would require many validated assays being available for accurate diagnosis.

VIRAL CULTURE

Given the diagnostic challenges due to Lassa virus diversity, viral isolation in cell culture remains the "gold standard" for the diagnosis of Lassa fever, although RT-PCR and immunoassays have become commonly used assays for a clinically actionable diagnosis. Viremia is often present at the time of presentation to medical care and declines after the sixth day of illness in patients who ultimately survived their infection, whereas it may persist until death in fatal cases (35). Culturing is performed by the inoculation of sample specimens suspected of containing the virus in Vero E6 cells at 37°C (36). A positive result may yield a cell cytopathic effect; however, a second method of detection, such as RT-PCR, viral antigen detection, or electron microscopy, must be used to confirm the identity of the virus (37). Lassa virus may be cultured from blood, throat swabs, urine, and cerebrospinal fluid samples from patients, although the detection of virus in throat swabs and urine is inconsistent among patients with serum viremia (35, 38, 39). The viral culture may be positive from organ samples (liver, spleen, lung, kidney, heart, and placenta) at autopsy in cases of fatal infections (35).

Viral culture allows for detection that is independent of genetic variations between strains and allows further characterization of the virus if desired. Viral culture also allows for the quantification of viremia, which may provide additional virus characterization information, as viremia with 10^3 50% tissue culture infective doses ($TCID_{50}$)/ml has a fatality odds ratio of 3.7 compared with viremia with less than 10^3 $TCID_{50}$/ml (35). Viral culture is neither rapid, taking at least several days to produce results, nor widely available due to the need for BSL-4 precautions to handle live viral specimens, which limits its utility for the early diagnosis of Lassa infection.

NUCLEIC ACID DETECTION METHODS

Real-time RT-PCR is a commonly used diagnostic approach for infectious diseases due to the high specificity and sensitivity of the method and has become a clinical standard for Lassa fever diagnosis. Coupled with automated sample processing and 96-well-plate-based thermocyclers, large numbers of samples can be tested quickly and inexpensively. PCR assays may be able to detect virus for a longer duration and earlier in illness compared with detection by viral culture (40) and may be performed on chemically inactivated specimens. The use of cycle threshold values with quantitative RT-PCR may assist with gross estimates of viremia (41) using appropriately selected positive-control material for standard curve generation. Depending on the primers and

virus strain used, the 95% probability limit of detection estimates with RT-PCR vary from 1,237 to 4,290 RNA copies/ml (29, 41, 42). However, with highly diverse pathogens such as Lassa virus, genetic diversity can be problematic for nucleic acid-based assays, as even a single nucleotide variant in one of the primers can have a significant negative impact on assay sensitivity depending on the location of the nucleotide variant (43).

Multiple real-time RT-PCR assays are published in the literature for Lassa virus (41, 44–46). For example, Safronetz and colleagues initially detected Lassa virus circulating in Mali using a SYBR green real-time RT-PCR assay targeting the Lassa virus S RNA segment (45), and Trombley and colleagues developed multiple probe-based Lassa virus assays due to strain diversity (44). However, standard RT-PCR assays are commonly used (28, 29, 46) due to their ease of use and the decreased specificity for probe-based real-time RT-PCR. Probe-based real-time RT-PCR (two primers and a probe) introduces the possibility of probe mismatches due to the high degree of Lassa virus diversity, potentially increasing the false-negative rate compared with that from RT-PCR using only two primers. While assays using DNA binding dyes (e.g., SYBR green real-time RT-PCR) are generally avoided due to the increased background observed due to primer, dimer, and target mispriming, such assays are advantageous when probe design is challenging or not possible (e.g., due to high diversity).

As additional sample testing and sequencing information becomes available, mismatches have been identified using established assays, necessitating assay redesign to improve performance. For example, Ölschläger and colleagues redesigned a commonly used standard RT-PCR assay for Lassa virus after identifying decreased assay sensitivity due to sequence variants for the reverse primer (29). This new RT-PCR assay is widely used for screening samples for Lassa virus and performed well in an external quality assessment study conducted by the European Network for Diagnostics of Imported Viral Diseases (46). Multiplex panels to simultaneously detect a multitude of viruses that can produce hemorrhagic fever syndromes, including Lassa and Ebola viruses, using RT-PCR alone or in combination with either enzyme hybridization or ligase detection reactions have also been developed (47–49).

ANTIGEN AND ANTIBODY DETECTION ASSAYS

Given the high diversity of the Lassa virus genome and the austere laboratory conditions where Lassa fever is endemic, antigen- and antibody-based assays are attractive alternatives to the high specificity and technical requirements of PCR assays. Antibody/antigen binding is generally less specific than primer/probe hybridization, allowing for greater flexibility in detecting diverse pathogens. Antigen detection relies on using specific antibodies against components of the Lassa virus to detect viral antigens in blood specimens. Initial assays detected nonspecific Lassa virus antigens with polyclonal antibodies, whereas more recent ELISAs target the Lassa virus nucleoprotein antigen. A diagnosis based on the detection of the relatively conserved Lassa nucleoprotein antigen (Fig. 1) may decrease the variability of test efficacy between genetically diverse viral strains in comparison to that from nucleic acid-based assays.

Lassa virus nucleoprotein antigen is detectable in patients with Lassa fever during the first week of illness and wanes during the second week in temporal association with the rise of detectable immunoglobulins (36). Increased levels of antigenemia have been found in fatal cases of Lassa fever compared with those in nonfatal cases (50). The short duration of antigenemia makes the detection of Lassa virus antigen more specific to acute Lassa virus infection than detection with antibody assays. Antigen detection assays may diagnose Lassa fever earlier during illness than antibody assays, as antibodies frequently may not be detectable until the second week of illness (36). However, Lassa virus antigen levels may become undetectable despite persistent viremia (51), and so a negative antigen test during an acute illness does not rule out Lassa fever.

Multiple antigen and IgM capture ELISAs have been developed using inactivated virus (36, 52–54); however this approach is limited to BSL-4 capable facilities. The use of recombinant antigens allows for improved assay development and access (50, 55–57). A lateral flow assay for Lassa virus nucleoprotein (ReLASV) is one type of rapid

diagnostic test that could be used for point-of-care testing. Following initial development and testing efforts (50, 57), this assay received the CE mark in 2013, although the assay has not been approved by the U.S. Federal Drug Administration. According to the product insert, the test yields results in 15 to 25 min and has 85% sensitivity and 99% specificity using confirmed Lassa virus-positive blood samples (Lassa virus positive by RT-PCR and IgM negative by ELISA) (58). In one study from Kenema Government Hospital in Sierra Leone, the use of ReLASV identified 95% of acute Lassa fever cases (defined as RT-PCR positive, increasing IgM titers, or IgM positive with IgG seroconversion), while missed cases were associated with resolving disease or mild disease with low levels of viremia (59).

Ideally, a diagnostic assay would not only detect Lassa virus infection but would also screen for multiple other pathogens with similar clinical presentations endemic in West Africa at the same time. Satterly and colleagues recently described transitioning Lassa and Ebola virus antigen- and IgM-based ELISAs onto a MAGPIX system (53) that uses individually labeled magnetic beads to detect multiple targets in a single assay. This assay has lower limits of detection for Lassa virus nucleoprotein and IgM than traditional ELISAs (53). The same group also developed and tested a multiplex MAGPIX IgG assay for a wide spectrum of hemorrhagic fever viruses, including alphaviruses, arenaviruses, flaviviruses, and filoviruses (14). Further development of multiplex MAGPIX assays, including testing for Lassa virus antigen and common endemic diseases such as malaria, would assist with the diagnosis and clinical management of suspected Lassa fever cases, especially in scenarios of coinfection with Lassa virus and bacterial or parasitic organisms where multiple therapeutic modalities may be indicated.

Delays in a patient seeking medical care following disease onset could negatively impact direct pathogen detection with a nucleoprotein detection assay, and diagnosis by Lassa virus-specific IgM may be more appropriate. Lassa IgM usually becomes detectable during the second week of infection (36), although it may be detectable within 4 days of onset of illness in some patients. A lack of an antibody response has been reported in some fatal cases of Lassa fever (60). Lassa virus IgG levels rise later than IgM levels, having a mean time to detection of 25.6 days after symptom onset (36), although positive IgG titers have occasionally been detected in patients with acute Lassa fever within the first few days of illness (35, 59).

Historically, antibody detection was conducted using immunofluorescence assays (IFAs) (4, 13, 36); however, IFAs have been replaced over time with ELISAs due to their ease of use, their increased sensitivity and specificity, and reduced interobserver variation in readings (36, 54). Estimates of the sensitivity of IgM detection for diagnosing Lassa fever compared with that of RT-PCR range from 55% to 72% (36, 61, 62). Antibody assays have been used to diagnose Lassa fever in those with a clinically consistent illness based on either detectable levels of Lassa virus antibody in a serum sample collected during illness or a rise in Lassa antibody titers (2, 63). However, Lassa virus IgM titers remain elevated for months to years following an acute infection (50). One study found that 28% of healthy hosts sampled from a region where the virus is endemic without a recent preceding febrile illness had detectable levels of Lassa virus IgM, suggesting that IgM positivity alone may be insufficient to diagnose Lassa fever in persons residing in regions where it is endemic (50). In regions where Lassa virus is endemic, a positive nucleoprotein antigen test, a rise in antibody titers between acute- and convalescent-phase serum, or the development of a new positive IgG titer in combination with a positive IgM titer may more accurately reflect acute Lassa fever than a single positive IgM test. While the detection of a new positive IgG titer combined with an IgM response in the correct clinical setting may support a diagnosis of Lassa fever, the detection of a positive IgG response alone is insufficient to make a diagnosis. Lassa IgG titers may persist for decades (64), and seroprevalence studies in regions of endemicity have shown 4 to 55% of healthy individuals living in areas where the virus is endemic have detectable Lassa virus IgG titers (14, 52, 55, 65, 66).

Bausch and colleagues conducted a direct comparison of acute Lassa fever cases by testing Lassa virus-positive samples identified by virus isolation and RT-PCR using IgM,

IgG, and antigen ELISAs and IFAs (36). Based on the onset of clinical symptoms, Lassa fever patients were generally antigen positive and IgM negative in the first week of clinical disease, were IgM positive and antigen negative in the second week, and were IgG positive around week 3 (36). The authors concluded that virus isolation is the most sensitive but clinically impractical diagnostic tool, and the combination of antigen capture assays and IgM ELISA was the best for diagnosis throughout the clinical disease course. However, direct pathogen detection allows the earliest diagnosis (when IgM is likely negative) and increases the chances of instituting ribavirin treatment in the first few days of illness when it is most efficacious.

SUMMARY AND CONCLUSIONS

Accurate and rapid diagnosis of Lassa fever is especially challenging due to the nonspecific clinical presentation, the high degree of Lassa virus genetic diversity observed in West Africa, and the biosafety concerns regarding laboratory testing for high consequence pathogens. While there are many diagnostic assays for Lassa virus, there currently is no timely, validated pan-Lassa virus assay available to both capture the diversity among viral strains and provide a diagnosis at any time point during the clinical course of illness.

Viral culture remains the "gold standard" for Lassa fever diagnosis across the diversity of Lassa strains but requires a clinically nonactionable amount of time and BSL-4 precautions to perform. Nucleic acid-based assays have become the clinical diagnostic standard and may be performed rapidly on inactivated specimens under BSL-2 conditions but may have false-negative results due to the high degree of genetic diversity among viruses. Viral antigen assays may provide a rapid diagnosis early on during illness but may miss the diagnosis at later stages once the antigenemia phase has resolved. The detection of a new IgM antibody response can diagnose Lassa fever but may miss the diagnosis during the first week of illness, may be falsely negative in severe infections where patients are unable to mount a serological response, and may remain positive for a prolonged period potentially causing false-positive results. A rise in baseline antibody titers between acute- and convalescent-phase serum or a positive IgM accompanied by the development of a new positive IgG response may be more indicative of acute Lassa fever in regions where it is endemic than a single positive IgM titer.

Overall, the appropriate diagnosis of Lassa fever will likely require a combination of a clinically compatible presentation along with serological and molecular diagnostic assays. Having a rapid, point-of-care multiplex test that can diagnose Lassa fever as well as other high consequence pathogens, such as Ebola virus, would accelerate accurate diagnosis, patient isolation, and efficacious therapy. As work for a Lassa virus vaccine and therapeutics moves toward clinical studies, having well-validated diagnostic assays available will be a necessity to ensure appropriate patient enrollment and countermeasure performance. Future directions for research in Lassa fever diagnostics should include assay improvement to increase detection across the genetically diverse spectrum of Lassa virus strains, assay validation to demonstrate efficacy across geographic regions and viral lineages, point-of-care diagnostic development and field validation, and content expansion of multiplex assays to distinguish Lassa fever from other diseases with similar clinical presentations.

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